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Identification of 50 Class D β-Lactamases and 65 *Acinetobacter*-Derived Cephalosporinases in *Acinetobacter* spp.

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Whole-genome sequencing of a collection of 103 *Acinetobacter* strains belonging to 22 validly named species and another 16 putative species allowed detection of genes for 50 new class D β-lactamases and 65 new *Acinetobacter*-derived cephalosporinases (ADC). All oxacillinases (OXA) contained the three typical motifs of class D β-lactamases, STFK, (F/Y)GN, and K(S/T)G. The phylogenetic tree drawn from the OXA sequences led to an increase in the number of OXA groups from 7 to 18. The topologies of the OXA and RpoB phylogenetic trees were similar, supporting the ancient acquisition of bla_{OXA} genes by *Acinetobacter* species. The class D β-lactamase genes appeared to be intrinsic to several species, such as *Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter calcoaceticus*, and *Acinetobacter lwoffii*. Neither $bla_{OXA-40/143}$- nor bla_{OXA-58}-like genes were detected, and their origin remains therefore unknown. The phylogenetic tree analysis based on the alignment of the sequences deduced from bla_{ADC} revealed five main clusters, one containing ADC belonging to species closely related to *A. baumannii* and the others composed of cephalosporinases from the remaining species. No indication of bla_{OXA} or bla_{ADC} transfer was observed between distantly related species, except for $bla_{OXA-279}$, possibly transferred from *Acinetobacter* genomic species 6 to *Acinetobacter parvus*. Analysis of β-lactam susceptibility of seven strains harboring new oxacillinases and cloning of the corresponding genes in *Escherichia coli* and in a susceptible *A. baumannii* strain indicated very weak hydrolysis of carbapenems. Overall, this study reveals a large pool of β-lactamases in different *Acinetobacter* spp., potentially transferable to pathogenic strains of the genus.

The genus *Acinetobacter* currently comprises 31 distinct species with valid names (http://www.bacterio.cict.fr/a/acinetobacter.html) and a number of taxa that include either species with effectively (but not validly) published names or genomic species delineated by DNA-DNA hybridization (1). Among them, *Acinetobacter baumannii* has emerged as the clinically and epidemiologically most important pathogen, essentially because of its ability to persist in the hospital environments and its propensity to acquire and develop antibiotic resistance mechanisms. *A. baumannii* is responsible for small epidemics of opportunistic infections, such as respiratory, bloodstream, urinary tract, skin, and soft tissue infections (1, 2). Two other species, *Acinetobacter pittii* and *Acinetobacter nosocomialis*, have also been implicated in nosocomial infections (3, 4). Together with *Acinetobacter calcoaceticus*, a soil organism, *A. baumannii*, *A. pittii*, and *A. nosocomialis* form the *A. calcoaceticus-A. baumannii* (ACB) complex (1). Two additional *Acinetobacter* genomic species (gen. spp.), provisionally termed "Between 1 and 3" and "Close to 13TU" have also been delineated within the ACB complex (4, 5). Other *Acinetobacter* species have been less frequently associated with clinical infections in humans, such as *Acinetobacter bereziniae*, *Acinetobacter guillouiae*, *Acinetobacter ursingii*, *Acinetobacter schindleri*, *Acinetobacter lwoffii*, *Acinetobacter parvus*, *Acinetobacter junii*, and *Acinetobacter johnsonii* (6–9).

In *A. baumannii*, a chromosomally encoded cephalosporinase, basal level expression of efflux pumps, and low membrane permeability are generally responsible for broad intrinsic resistance. The presence of intrinsic or acquired β-lactamases is mainly responsible for resistance to β-lactams. Historical classifications divide β-lactamases into classes A to D (10–12), in which A, C, and D are serine-active β-lactamases whereas B is composed of metallo-β-lactamases (13). Carbapenems, often used against Gram-negative bacteria, are considered antibiotics of last resort against multidrug-resistant strains of *A. baumannii*. Resistance to these drugs is thus a serious concern and is mainly due to production of carbapenemases that belong to β-lactamases of class A, B, or D, with a high prevalence, in *A. baumannii*, of class D β-lactamases, also called OXA-type β-lactamases (14, 15). This species carries genes encoding two intrinsic β-lactamases: an AmpC-type cephalosporinase encoded by bla_{ADC} termed ADC (*Acinetobacter*-derived cephalosporinase) and an OXA-51-type β-lactamase. These two enzymes confer a very low level β-lactam resistance. However, the presence of an insertion sequence, such as IS*Aba1* or IS*Aba9*, upstream from bla_{ADC} or bla_{OXA-51} can be responsible, by bringing a strong promoter, for an increase in expression of these genes and thus reduction in susceptibility to β-lactams (16–19).

Recently, detection of new OXA-type β-lactamases has dramatically increased worldwide; as many as 208 OXA-type β-lactamases were reported in 2012 (20), and in September 2013, 365 OXAs enzymes were known (http://www.lahey.org/Studies/other.asp#table 1). These β-lactamases could be divided in seven groups (OXA-2, OXA-10, OXA-23, OXA-24/33/40, OXA-51,

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OXA-58, and OXA-134), of which five (OXA-23, OXA-24/33/40, OXA-51, OXA-58, and OXA-134) contained enzymes designated carbapenem-hydrolyzing class D β-lactamases (CHDL), while the two remaining groups are represented by narrow-spectrum oxacillinases OXA-2 and OXA-10 (20).

OXA-51 is considered a naturally occurring CHDL in *A. baumannii* and OXA-134 as intrinsic to *A. lwoffii* and *A. schindleri* (21, 22), whereas OXA-23, OXA-24/33/40, and OXA-58 are classified as acquired CHDLs. Proteins of these five groups share only from 47 to 62% identity (20), suggesting that they could originate from various reservoirs. It has been proposed that *A. radioresistens* is the source of the bla_{OXA-23} gene (23).

The aim of this study was to identify, by a whole-genome sequencing approach, OXA- and ADC-type β-lactamases and to search for the origin of $bla_{OXA-40/-143}$ and bla_{OXA-58} in a collection of 103 strains representative of the diversity of the genus *Acinetobacter*.

MATERIALS AND METHODS

Bacterial strains. A total of 103 *Acinetobacter* strains obtained from the collections of A. Nemec (strains designated NIPH or ANC) or the Institut Pasteur (designated CIP) were included in the present study (Table 1). These strains were selected to reflect the currently known breadth of the diversity of the genus *Acinetobacter* at the species level. Ninety-nine of the 103 strains belonged to 22 validly named species, to 8 genomic species (gen. spp.) as defined by Bouvet and Grimont (24, 25), Bouvet and Jeanjean (26), Tjernberg and Ursing (27), and Gerner-Smidt and Tjernberg (5), or to 8 tentative novel species termed *Acinetobacter* taxons 18 to 23, 25, and 26. The four remaining strains were taxonomically unique, i.e., not belonging to any of the listed species or taxa. The *Acinetobacter* taxa are working taxonomic groups as delineated at the Laboratory of Bacterial Genetics (National Institute of Public Heath, Prague, Czech Republic) based on comprehensive physiological/nutritional testing, *rpoB* and 16S rRNA gene comparative analyses, and whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) profiling (8). Each of these taxa is, at the species level, clearly distinct from any of the known species with valid names, genomic species, or species with effectively published names (except for taxon 25, which is close to *A. brisouii*). Each of the four unique strains was close to (but distinct from, at the genomic species level) one of the species/taxa; these strains are termed *A. pittii*-like (ANC 4050 and ANC 4052), *A. calcoaceticus*-like (ANC 3811), or taxon 18-like (NIPH 284) in this study. Organisms were grown, according to their physiological requirements, at 30 to 37°C in brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, MI).

Antibiotic susceptibility testing. MICs were determined by the Etest procedure (bioMérieux, Marcy l'Etoile, France) on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France).

DNA manipulations. *A. baumannii* genomic DNA was extracted as described previously (28). DNA amplification was performed in a GeneAmp PCR system 9700 (Perkin-Elmer Cetus, Norwalk, CT) with Phusion DNA polymerase (Finnzymes, Espoo, Finland). Amplification of bla_{OXA} genes from *A. parvus*, *A. pittii*, *A. pittii*-like, *Acinetobacter* gen. sp. "Between 1 and 3," *A. bereziniae*, *A. venetianus*, and *Acinetobacter* gen. sp. 6 were performed using specific primers (Table 2). Due to the high similarity of $bla_{OXA-273}$ and $bla_{OXA-270}$ and of $bla_{OXA-279}$ and $bla_{OXA-290}$, the same pair of primers was used to amplify two genes. PCR elongation times and temperatures were adjusted according to the expected size of the PCR products and the nucleotide sequence of the primers. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA). DNA fragments were extracted from agarose gels with a QIAquick gel extraction kit (Qiagen). Nucleotide sequencing was carried out with a CEQ 8000 DNA analysis system automatic sequencer (Beckman Instruments, Inc., Palo Alto, CA).

Recombinant DNA techniques. DNA isolation, digestion with restriction endonucleases (New England BioLabs, Ipswich, MA), ligation with T4 DNA ligase (New England BioLabs), and transformation of competent *Escherichia coli* TOP10 (Invitrogen, San Diego, CA) with recombinant plasmid DNA were performed by standard methods (28). PCR amplification products of $bla_{OXA-279}$, $bla_{OXA-290}$, $bla_{OXA-273}$, $bla_{OXA-270}$, $bla_{OXA-304}$, $bla_{OXA-301}$, and $bla_{OXA-266}$ were cloned in pCRblunt (Invitrogen) and transformed into TOP10 cells with selection on BHI-agar plates containing kanamycin (50 mg/liter).

Plasmid pCM88 containing bla_{OXA-23} was a gift of T. Lambert (unpublished data). Briefly, the bla_{OXA-23} gene and its regulatory region, in which the −35 sequence TAACTA was replaced by the RNA polymerase TTGACA consensus motif for the σ^{70} factor of *E. coli*, and the replication origin of pWH1266 (GenBank accession number M36473) were cloned in pUC18gm (29) to give pCM88. Plasmids pCM88Δbla_{OXA-23}Ω$bla_{OXA-279}$ and pCM88Δbla_{OXA-23}Ω$bla_{OXA-290}$ were constructed from pCM88 by replacement of the insert. In a first PCR, $bla_{OXA-279}$ was amplified from pCRbluntΩ$bla_{OXA-279}$ using primers OXA-ParGsp6RBS, 5′*ctattcatctggtgtttaaa*ATGCCAAAAATACTAAAACATCTT3′ (italicized nucleotides, containing the ribosome binding site [RBS] of bla_{OXA-23}, are complementary to 20 nucleotides [nt] of primer PCM88rev), and OXA-ParGsp6XbaI, 5′-CTAG*TCTAGA*TTATTGCGTCTCTAACAATTTCAA-3′ (where the XbaI site is italicized). A second PCR allowed obtainment of a 218-bp fragment containing the promoter and the RBS of bla_{OXA-23} using pCM88 as a template and primers pCM88for, 5′AGTGAGCGCAACGCAATTAATGTG3′, and pCM88rev, 5′CAT*tttaaacaccagatgaatag*3′ (italicized nucleotides are complementary to the first 20 nucleotides of primer OXA-ParGsp6RBS). The two PCR products were then linked by overlapping PCR with primers PCM88UpR1/OXA-ParGsp6XbaI, generating a 1,065-bp fragment that was digested by EcoRI and XbaI, ligated to EcoRI-XbaI-linearized pCM88 in place of bla_{OXA-23}, and introduced by electrotransformation into competent susceptible *A. baumannii* BM4587 (30) with selection on BHI plates containing gentamicin (20 mg/liter). Plasmid pCM88Δbla_{OXA-23}Ω$bla_{OXA-290}$ was constructed similarly using the same primers (OXA-ParGsp6RBS and OXA-ParGsp6XbaI) for the initial PCR.

Library construction and sequencing. Illumina fragment libraries were generated as previously described (31) with the following modifications. For each sample, 100 ng of genomic DNA was sheared to 150 to 300 bp in size using a Covaris LE220 instrument (Covaris) with the following parameters: temperature, 7 to 9°C; duty cycle, 20%; intensity, 5; number of cycles per burst, 200; time, 90 s; shearing tubes, Crimp-Cap microTubes with AFA fibers (Covaris). DNA fragments were end repaired, 3′ adenylated, ligated with indexed Illumina sequencing adapter, and PCR enriched as previously described (32). The resulting Illumina fragment sequencing libraries were size selected to contain inserts of 180 bp ± 3% in length using a Pippen Prep system (Sage Science) according to the manufacturer's recommendations. Illumina 3-kb jumping sequencing libraries were prepared according to the manufacturer's protocol (2- to 5-kb insert Illumina Mate-pair library prep kit V2; Illumina) with the following modifications. For each sample, 5 μg of genomic DNA was sheared to 3 to 5 kb in size using a Covaris LE220 instrument (Covaris) with the following parameters: temperature, 19 to 21°C; duty cycle, 20%; intensity, 2; number of cycles per burst, 1,000; time, 300 s; shearing tubes, MiniTube-red (Covaris). Fragmented DNA was size selected using 0.45 volume of Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's recommendations. DNA fragments were end repaired using NEBNext Reagents for DNA Sample Preparation for Illumina (New England BioLabs) and biotinylated deoxynucleoside triphosphates (dNTPs) (Perkin-Elmer) according to the manufacturer's recommendations. Blunt-ended, biotinylated DNA fragments were circularized at 16°C using T3 DNA Ligase (Enzymatics). Following ligation, remaining linear fragments were removed by exonuclease treatment using PlasmidSafe ATP-Dependent DNase (Epicentre Biotechnologies). Circularized fragments were sheared to ~450 bp in length using a Covaris LE220 instrument with the following parameters: temperature, 7°C; duty cycle, 15%;

TABLE 1 Oxacillinases and *Acinetobacter*-derived cephalosporinases detected in this study

Species	No. of strains	Strain	Reference(s)	OXA[a]	ADC[a]
A. baumannii	21	ANC 4097	41	OXA-69	ADC-11
		CIP 70.34^T	4, 24	OXA-98	**ADC-CIP 70–34^T**
		NIPH 1362	39, 42	**OXA-260**	**ADC-NIPH 1362**
		NIPH 146	4, 43	OXA-64	**ADC-NIPH 146**
		NIPH 1669 (= LUH 5875)	4	OXA-71	**ADC-NIPH 1669**
		NIPH 1734	4, 42	OXA-51	**ADC-NIPH 1734**
		NIPH 190	4, 42	OXA-65	**ADC-NIPH 190**
		NIPH 201	4, 42	**OXA-261**	**ADC-NIPH 201**
		NIPH 2061	This study	OXA-109	**ADC-NIPH 2061**
		NIPH 24	39, 42	OXA-66	**ADC-NIPH 528**
		NIPH 290	42	OXA-69	ADC-11
		NIPH 329	4, 42	**OXA-263**	**ADC-NIPH 329**
		NIPH 335	4, 42	OXA-128	**ADC-NIPH 335**
		NIPH 527 (= RUH 875)	4, 44	OXA-69	ADC-11
		NIPH 528 (= RUH 134)	4, 44	OXA-66	**ADC-NIPH 528**
		NIPH 60	4, 42	OXA-69	**ADC-NIPH 60**
		NIPH 601	4, 42	OXA-69	**ADC-NIPH 601**
		NIPH 615	4, 42	OXA-120	**ADC-NIPH 615**
		NIPH 67	4, 42	**OXA-262**	**ADC-NIPH 67**
		NIPH 70	4, 42	**OXA-259**	**ADC-NIPH 146**
		NIPH 80	4, 42	OXA-78	**ADC-NIPH 80**
A. baylyi	1	CIP 107474^T	45		**ADC-CIP 107474^T**
A. beijerinckii	2	CIP 110307^T	27, 46		**ADC-CIP 110307^T**
		ANC 3835	This study		**ADC-ANC 3835**
A. bereziniae	2	CIP 70.12^T	8, 24	**OXA-301**	**ADC-CIP 70–12^T**
		NIPH 3	8	**OXA-300**	**ADC-NIPH 3**
A. bouvetii	1	CIP 107468^T	45	**OXA-299**	
A. brisouii	1	ANC 4119^T (= CCUG 61636^T)	47		
A. calcoaceticus	3	CIP 81.8^T	4, 24	OXA-213	**ADC-CIP 81–8^T**
		NIPH 13	4, 43	**OXA-268**	**ADC-NIPH 13**
		ANC 3680	4	**OXA-267**	**ADC-ANC 3680**
A. gerneri	1	CIP 107464^T	45	**OXA-308**	
A. guillouiae	2	CIP 63.46^T	8, 24	**OXA-274**	**ADC-CIP 63–46^T**
		NIPH 991	8	**OXA-275**	**ADC-NIPH 991**
A. haemolyticus	2	CIP 64.3^T	24	**OXA-264**	**ADC-CIP 64–3^T**
		NIPH 261	48	**OXA-265**	**ADC-NIPH 261**
A. johnsonii	2	CIP 64.6^T	24	**OXA-281**	
		ANC 3681	This study	**OXA-280**	**ADC-ANC 3681**
A. junii	3	CIP 64.5^T	24		**ADC-CIP 64–5^T**
		CIP 107470[b]	45		**ADC-CIP 107470**
		NIPH 182	48		**ADC-NIPH 182**
A. lwoffii	8	CIP 101966	This study	**OXA-283**	
		CIP 102136	This study	OXA-134	
		CIP 51.11	This study	**OXA-284**	
		CIP 64.7	24	**OXA-285**	
		CIP A162	24	**OXA-283**	
		CIP 70.31	24	OXA-134	
		NIPH 478	48	Partial	
		NIPH 715	48	**OXA-282**	
A. nosocomialis	2	NIPH 386	4		**ADC-NIPH 386**
		NIPH 2119^T	4		**ADC-NIPH 2119^T**
A. parvus	8	CIP 108168^T	7	**OXA-279**	
		CIP 102082	This study	**OXA-279**	
		CIP 102129	This study		
		CIP 102143	This study	**OXA-279**	
		CIP 102159	This study	**OXA-279**	
		CIP 102529	This study	**OXA-279**	
		CIP 102637	This study		
		NIPH 1103 (= LMG 21766)	7		
A. pittii	2	CIP 70.29^T	4, 24	**OXA-272**	ADC-18
		ANC 3678	4	**OXA-273**	**ADC-ANC 3678**

(Continued on following page)

TABLE 1 (Continued)

Species	No. of strains	Strain	Reference(s)	OXA[a]	ADC[a]
A. radioresistens	2	CIP 103788^T	49	OXA-103	
		NIPH 2130 (= SEIP 12.8)	24	OXA-23	
A. schindleri	3	CIP 107287^T	6	**OXA-277**	
		CIP 101934	This study	**OXA-278**	
		NIPH 900	6	**OXA-276**	
A. soli	2	CIP 110264^T	50		
		NIPH 2899	This study		
A. towneri	1	CIP 107472^T	45		
A. ursingii	3	CIP 107286^T	6		**ADC-CIP 107286^T**
		ANC 3649 (= CCUG 56015)	51		**ADC-ANC 3649**
		NIPH 706	6		**ADC-NIPH 706**
A. venetianus	1	CIP 110063^T	52	**OXA-266**	**ADC-CIP 110063^T**
Gen. sp. 6	2	CIP A165	24	**OXA-290**	**ADC-CIP A165**
		NIPH 298	48	**OXA-289**	**ADC-NIPH 298**
Gen. sp. 13BJ/14TU	2	NIPH 1859 (= 71)	27	**OXA-307**	**ADC-NIPH 1859**
		CIP 64.2	26	**OXA-302**	**ADC-CIP 64–2**
Gen. sp. 14BJ	2	NIPH 1847 (= 382)	26	**OXA-297**	**ADC-NIPH 1847**
		ANC 3623	This study	**OXA-298**	**ADC-ANC 3623**
Gen. sp. 15TU	2	NIPH 899	48		**ADC-NIPH 899**
		NIPH 2171 (= 151a)	27		
Gen. sp. 16	3	ANC 3880	This study	**OXA-303**	**ADC-ANC 3880**
		CIP 56.2	This study	**OXA-287**	**ADC-CIP 56–2**
		CIP 70.18	26	**OXA-288**	**ADC-CIP 70–18**
Gen. sp. 17	2	ANC 4105	This study	**OXA-291**	**ADC-ANC 4105**
		NIPH 1867 (= SEIP Ac87.314)	26	**OXA-292**	**ADC-NIPH 1867**
Gen. sp. "Close to 13TU"	1	NIPH 973 (= 10090)	4, 5		**ADC-NIPH 973**
Gen. sp. "Between 1 and 3"	2	NIPH 542 (= 10169)	4, 5	**OXA-304**	**ADC-NIPH 542**
		NIPH 817 (= 10095)	4, 5	**OXA-305**	**ADC-NIPH 817**
Taxon 18	2	ANC 3862	This study		**ADC-ANC 3862**
		NIPH 236	48		**ADC-NIPH 236**
Taxon 19	1	NIPH 809 (= CCUG 14818)	26	**OXA-286**	**ADC-NIPH 809**
Taxon 20	2	NIPH 758	This study	**OXA-294**	**ADC-NIPH 758**
		NIPH 2168 (= 1240)	26	**OXA-295**	**ADC-NIPH 2168**
Taxon 21	1	ANC 3929	This study	**OXA-293**	**ADC-NIPH 3929**
Taxon 22	1	NIPH 2100 (= SEIP 1/87)	26	**OXA-306**	**ADC-NIPH 2100**
Taxon 23	1	NIPH 713	48		
Taxon 25	1	ANC 3789	This study		
Taxon 26	1	ANC 3994	This study	**OXA-296**	**ADC-ANC 3994**
A. calcoaceticus-like	1	ANC 3811	This study	**OXA-269**	**ADC-ANC 3811**
A. pittii-like	2	ANC 4050	This study	**OXA-270**	**ADC-ANC 4050**
		ANC 4052	This study	**OXA-271**	**ADC-ANC 4052**
Taxon 18-like	1	NIPH 284	48		Partial

[a] New β-lactamases are indicated in bold.
[b] Type strain of *A. grimontii*, a junior synonym of *A. junii*.

TABLE 2 Oligodeoxynucleotides

PCR target	Primer	Sequence (5′→3′)	Positions[a]
OXA-279/ OXA-290	OXA-pa-F	ATGCCAAAAATACTAAAACA	1–20
	OXA-pa-R	TTATTGCGTCTCTAACAATT	840–821
OXA-273/ OXA-270	OXA-pi-F	ATGACTAAAAAAGCTCTTTT	1–20
	OXA-pi-R	CTATAAAATACCGAGTTGT	822–804
OXA-304	OXA-b1-3-F	ATGTATAAAAAAGTCCTTG	1–19
	OXA-b1-3-R	CTATAAAATACCGAGTTGT	822–804
OXA-266	OXA-vene-F	ATGCTAAAAAAGTTCAAAG	1–19
	OXA-vene-R	CTAATGCTGAATTAAACCT	834–816
OXA-301	OXA-bere-F	ATGAAGTTTAAAATGAAAGG	1–20
	OXA-bere-R	TAATCTTAAATAAAAAAATA	831–812

[a] Coordinates refer to the first base of the gene.

intensity, 10; number of cycles per burst, 200; time, 90 s; shearing tubes, Crimp-Cap microTubes with AFA fibers (Covaris). Sheared fragments were size selected using 0.65 volume of Agencourt AMPure XP beads (Beckman Coulter Genomics) and immobilized using Dynal M280 streptavidin-coupled Dynabeads 1 of 7 (Invitrogen) according to the manufacturer's recommendations. Illumina sequencing libraries were prepared from immobilized fragments using the NEBNext Reagents for DNA Sample Preparation for Illumina according to the manufacturer's suggested protocol (New England BioLabs) using indexed Illumina sequencing adapters. Libraries were enriched according to the Illumina protocol and purified using 0.65 volume of Agencourt AMPure XP beads (Beckman Coulter Genomics). All liquid handling steps were performed using a Bravo Automated Liquid Handler (Agilent Technologies). Illumina sequencing libraries were quantified using quantitative PCR (qPCR; KAPA Biosystems) according to the manufacturer's recommendations. Libraries were normalized to 2 nM and denatured using 0.1 N NaOH. Sequencing Flowcell cluster amplification was performed according to the manufactur-

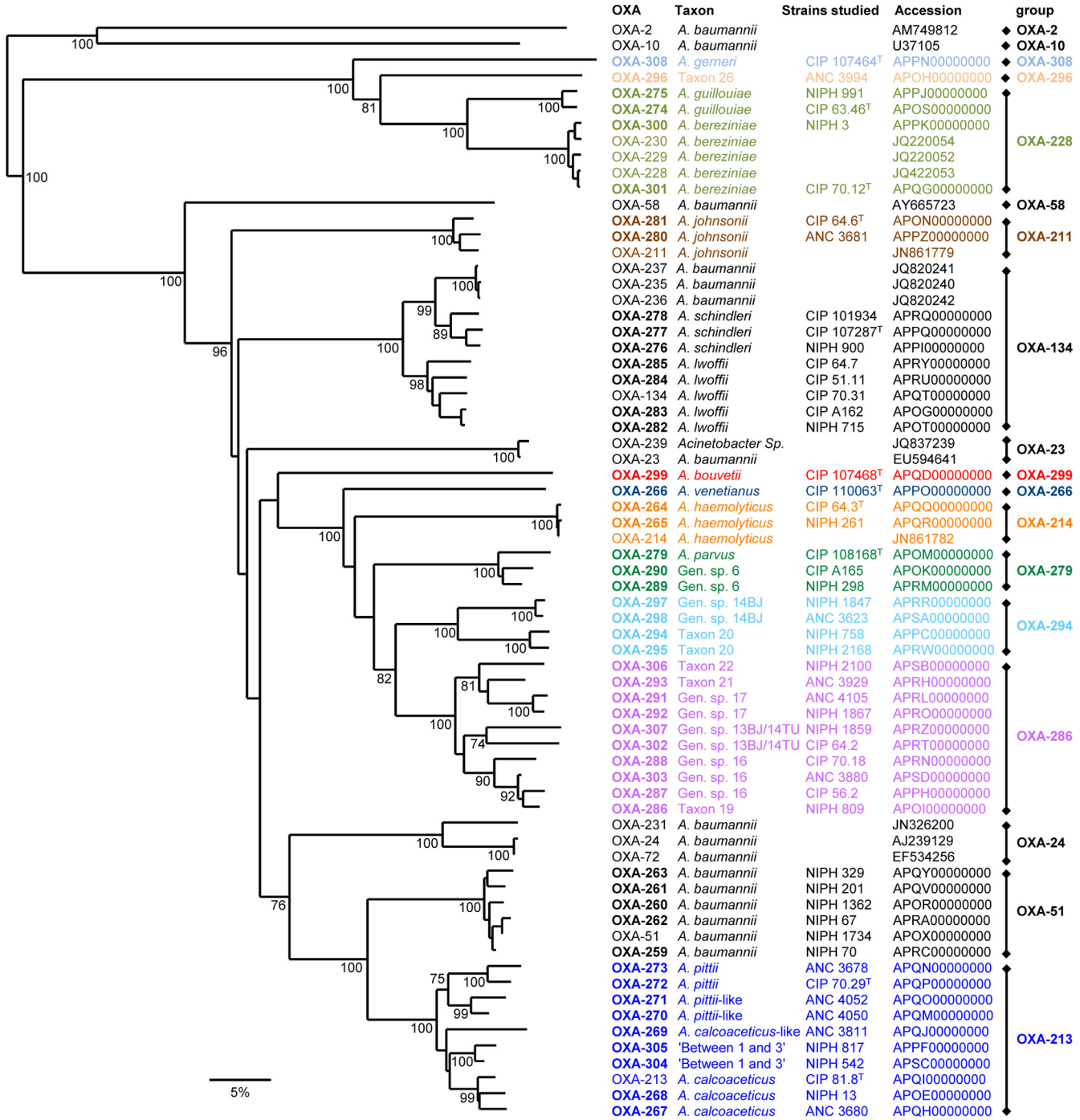

FIG 1 Neighbor-joining phylogenetic tree obtained for 69 OXA-like class D β-lactamases. The classification in 18 main groups is indicated on the right. Proteins from the same group exhibit at least 80% identity. The accession numbers of the genome sequences from which the data were retrieved are indicated in the last column. New OXAs are indicated in bold. Bootstrap values (≥70%) obtained after 1,000 replicates are given at the nodes. Bar, 5% sequence divergence.

er's recommendations using the V3 TruSeq PE Cluster kit and V3 TruSeq Flowcells (Illumina). Flowcells were sequenced with 101 base-paired end reads on an Illumina HiSeq2000 instrument, using V3 TruSeq Sequencing-by-Synthesis kits and analyzed with the Illumina RTA v1.12 pipeline.

Genome assembly and annotation. Assemblies were performed by Allpaths-LG (33) with default parameters except for the genomes of *A. baumannii* NIPH 2061, NIPH 329, NIPH 601, NIPH 528, NIPH 70, ANC 4097, NIPH 527, and *A. pittii*-like ANC 4052, which were reference assisted with the *A. baumannii* AYE genome sequence and the Assisted_Patching = 2 parameter. Genomes were analyzed using the Gamer analysis package, and any contigs we deemed to be contamination were removed from the assembly. Predictions of protein-coding genes (open reading frames [ORFs]) were made by Prodigal (34). rRNA genes were predicted by RNAmmer (35) and tRNAs by tRNAscan-SE (36). ORFs having more than 70% overlap with either tRNA or rRNA genes were disregarded. Gene product names were assigned based on BLAST best hit

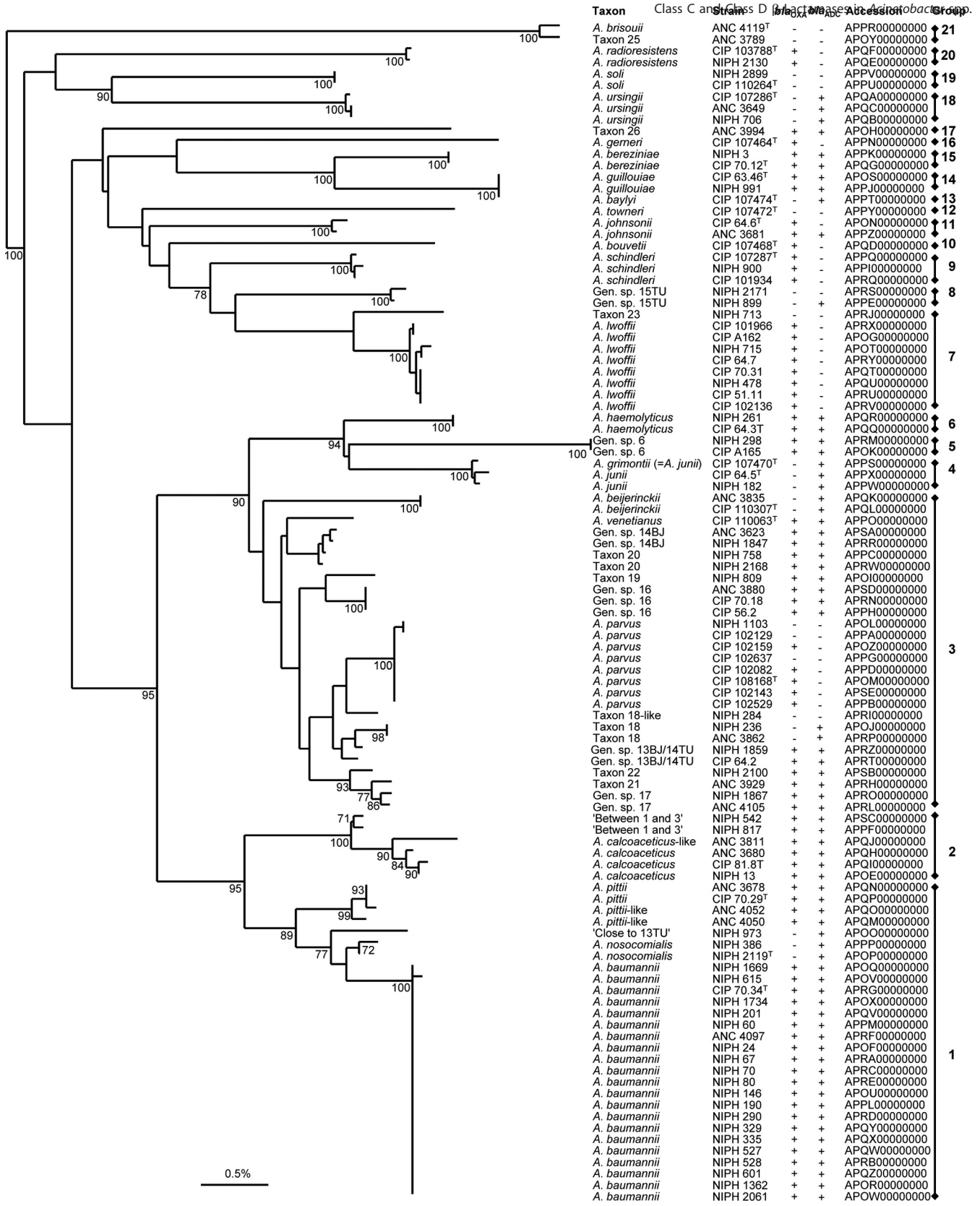

FIG 2 Phylogenetic tree obtained for RpoB protein sequences inferred from 103 whole-genome sequences obtained in this study. The classification into 16 groups and 5 singletons is indicated on the right. Each group includes a cluster of proteins with mutual sequence identity of 98% or more. The high similarity of RpoB proteins derived from *A. guillouiae* and *A. baylyi* results from the transfer of the complete *rpoB* gene from *A. guillouiae* to *A. baylyi* CIP 107474^T (8) and does not indicate the close relatedness of these two species. The sequences were compared using the neighbor-joining method and a simple matching cost matrix. The accession numbers of the genome sequences from which the data were retrieved are indicated in the last column. Bootstrap values (≥70%) obtained after 1,000 replicates are given at the nodes. Bar, 0.5% sequence divergence.

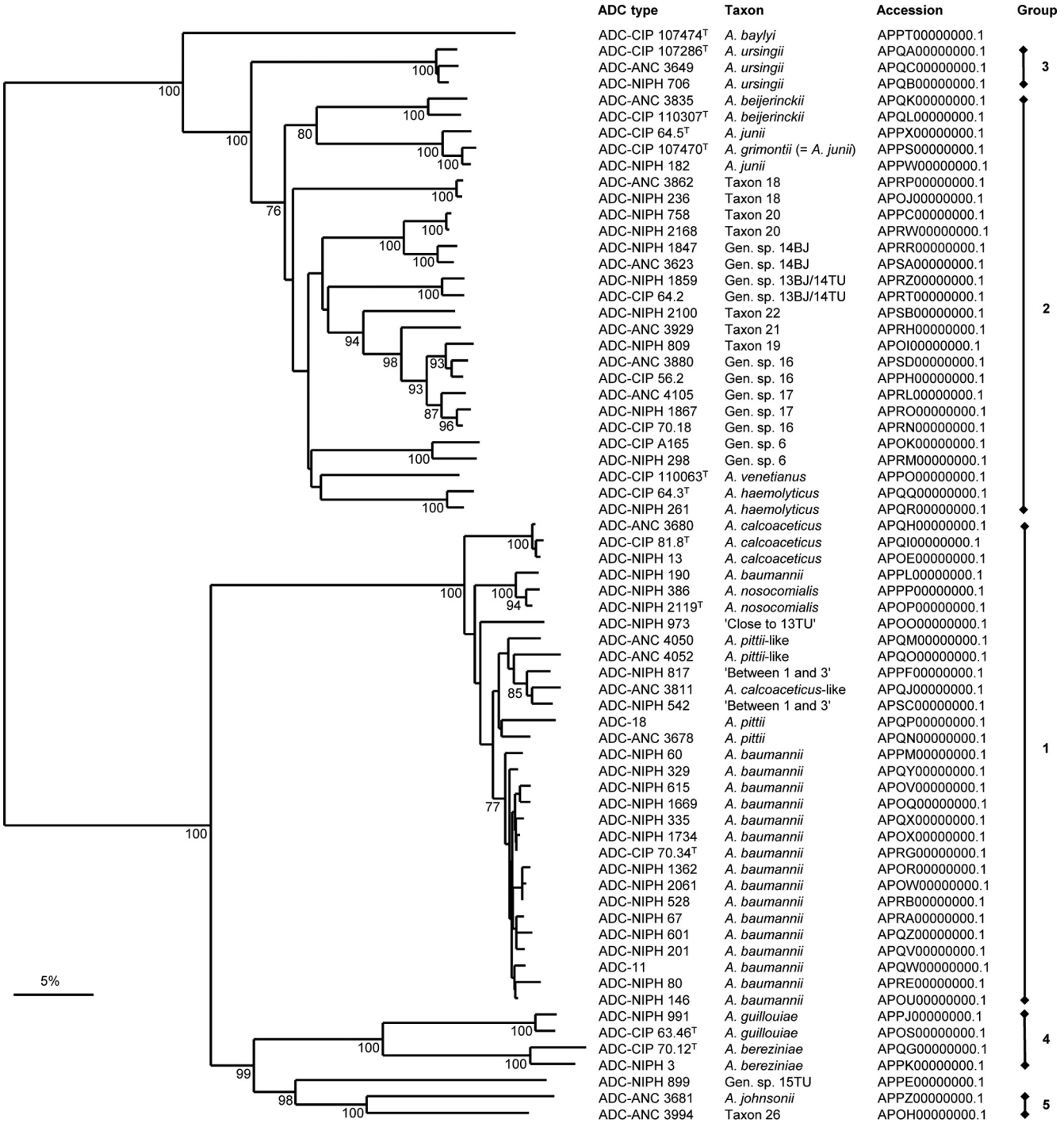

FIG 3 Neighbor-joining phylogenetic tree based on the protein sequences of 66 *Acinetobacter*-derived cephalosporinases inferred from the whole-genome sequences obtained in this study. Clusters encompassing proteins with sequence identity of at least 77% are indicated on the right. Novel ADC variants are designated with the number of the host strain. The accession numbers of the genome sequences from which the data were retrieved are indicated in the last column. Bootstrap values (≥70%) obtained after 1,000 replicates are given at the nodes. Bar, 5% sequence divergence.

to the Swiss-Prot database (37) (sequence identity, ≥70%; query coverage, ≥70%). The remainder was annotated via hidden Markov model (HMM) profile alignment to TIGRfam equivalogs and finally based on BLAST best hits to the KEGG database (38) protein sequences with KO numbers (sequence identity, ≥50%; query coverage, ≥50%). The genes that did not match any known gene in the Swiss-Prot and KEGG databases were considered to encode hypothetical proteins.

OXA numbering. OXA numbers were assigned by the Lahey clinic according to the sequences in their database (http://www.lahey.org/Studies/).

Comparative sequence analyses. The complete sequences of the OXA-like, ADC-like, and RpoB proteins inferred from whole-genome sequences obtained in this work or retrieved from the EMBL library were analyzed using Bionumerics 6.6 software (Applied-Maths, St-Martens-

TABLE 3 Percent identity between and within OXA groups[a]

Group	Group																
	OXA -51	**OXA -213**	**OXA -23**	**OXA -24**	**OXA -134**	**OXA -211**	**OXA -294**	**OXA -286**	**OXA -214**	**OXA -279**	**OXA -228**	**OXA -2**	**OXA -10**	**OXA -58**	**OXA -308**	**OXA -266**	**OXA -299**
OXA-51	**94-99**																
OXA-213	77	**84-97**															
OXA-23	60	62	**97-99**														
OXA-24	66	66	64	**87-99**													
OXA-134	58	59	61	57	**86-99**												
OXA-211	61	61	60	60	61	**95-96**											
OXA-294	59	58	54-56	57-58	56-58	58-59	**84-98**										
OXA-286	59-60	57-59	54-55	58-59	57-59	55-56	74-76	**82-97**									
OXA-214	59	59	55	56	55	57	69-70	68-69	**99**								
OXA-279	59	58-59	54-55	57-58	58	53	71-75	68-72	68	**93-95**							
OXA-228	56-57	61	56	57	61	57	60-62	60-61	58	61-62	**81-99**						
OXA-2	28	28	27	28	29	29	29-30	28-29	30	29	31	**80-99**					
OXA-10	35	36	37	39	35-36	39	36-37	35-36	35	35-36	37	35	**NA**[a]				
OXA-58	51	50	50	51	53-54	56	53-54	51-53	52	51-53	57-59	30	37	**99**			
OXA-308	55	57	54	55	52-53	56	55	56	54	54-56	65	27	36	54	**NA**		
OXA-266	58	58	59	56	58-60	58	68-70	69-71	66	69-70	62	27	37	53	56	**NA**	
OXA-299	60	61	56	57	54	57	58	58-59	59	56-57	58	27	35	49	53	57	**NA**
OXA-296	56	55	53	53	54-56	52	55-56	54-55	54	55-56	67	25	32	58	59	57	54

[a] Identity within an OXA group is indicated in bold. NA, not applicable (only a single sequence was available).

Latem, Belgium) with default parameters (an open gap penalty of 100%, a unit gap penalty of 0%, enabling fast algorithm for alignment calculations; a gap penalty of 0% for similarity calculations). Calculations were based on a simple matching cost matrix with similarity values expressed as the percentages of identical amino acid residues in corresponding positions in two aligned sequences, while the neighbor-joining method was used for clustering.

Multilocus sequence analysis (MLSA) calculations were based on the internal portions of seven protein-encoding housekeeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*), which are included in the *A. baumannii* multilocus sequence analysis scheme described by Diancourt et al. (39) (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html). Phylogenetic analysis of concatenated sequences (2,976 nt in total) was performed with the Bionumerics 6.6 software using the neighbor-joining method based on the Jukes-Cantor model of evolution.

Nucleotide sequence accession numbers. The accession numbers for whole-genome sequences, including those determined in this study, are indicated in Fig. 1, 2, and 3.

RESULTS AND DISCUSSION

Detection of oxacillinase genes. Whole-genome sequencing, performed on all of the 103 *Acinetobacter* strains (Table 1), allowed to characterize class D β-lactamases in 77 strains belonging to 13 named *Acinetobacter* spp., six genomic species, and five taxa or remained unallocated to any species/taxon. As expected, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-134-like}}$, $bla_{\text{OXA-211-like}}$, $bla_{\text{OXA-213-like}}$, $bla_{\text{OXA-214-like}}$, and $bla_{\text{OXA-228-like}}$ were found in *A. radioresistens*, *A. baumannii*, *A. lwoffii*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus*, and *A. bereziniae*, respectively. Genes encoding as many as 50 new OXAs were found in 55 strains (Table 1).

Deduced sequences from all bla_{OXA} revealed 273- to 280-amino-acid proteins containing the typical motifs of class D β-lactamases: STFK (positions 70 to 73), (F/Y)GN (positions 144 to 146), and K(S/T)G (positions 216 to 218) (see Fig. S1 in the supplemental material). BLASTTP analysis indicated that the new oxacillinases exhibited from 49 to 95% identity with already described CHDLs.

The oxacillinase in *A. bereziniae* CIP 70.12^{T} has been previously described as OXA-228 (40). However, the amino acid sequence derived from our genome sequence of CIP 70.12^{T}, identical to that of *A. bereziniae* LMG 1003^{T} contig 0035 (GenBank accession no. AIEI01000035), differed from OXA-228 by $V_{36}E$.

OXA-266 from *A. venetianus* CIP 110063^{T} had 99% identity with a class D β-lactamase found in *Acinetobacter* sp. NBRC

TABLE 4 Relation between the OXA and RpoB groups

OXA group	OXA no.	Species	RpoB group
51[a]	**259, 260, 261, 262, 263**	*A. baumannii*	1
213[a]	**272, 273**	*A. pittii*	1
	270, 271	*A. pittii* like	1
	304, 305	Gen. sp. "between 1 and 3"	2
	267, 268	*A. calcoaceticus*	2
	269	*A. calcoaceticus* like	2
266[b]	**266**	*A. venetianus*	3
286[b]	306	Taxon 22	3
	291,292	Gen. sp. 17	3
	293	Taxon 21	3
	287, 288, 303	Gen. sp. 16	3
	286	Taxon 19	3
	302, 307	Gen. sp. 13BJ/14TU	3
294[b]	297, 298	Gen. sp. 14BJ	3
	294, 295	Taxon 20	3
279[b]	279	*A. parvus*	3
	289, 290	Gen. sp. 6	5
214	264, 265	*A. haemolyticus*	6
134	**282, 283, 284, 285**	*A. lwoffii*	7
	276, 277, 278	*A. schindleri*	9
299	**299**	*A. bouvetii*	10
211	**280, 281**	*A. johnsonii*	11
228	274, 275	*A. guillouiae*	14
	300, 301	*A. bereziniae*	15
308	308	*A. gerneri*	16
296	296	Taxon 26	17

[a] OXA-213 and OXA-51 shared 77% identity.
[b] The four OXA groups OXA-266, OXA-286, OXA-294, and OXA-279 shared between 68% and 76% identity.

TABLE 5 Antibiotic susceptibility of *Acinetobacter* strains

Antibiotic	MIC[a] (mg/liter) for strain:						
	A. parvus (CIP 108168^T)	Gen. sp. 6 (CIP A165)	*A. pittii* (ANC 3678)	*A. pittii*-like (NIPH 4050)	Gen. sp. "Between 1 and 3" (NIPH 542)	*A. bereziniae* (CIP 70.12^T)	*A. venetianus* (CIP 110063^T)
Amoxicillin	2	1	48	32	16	8	6
Amoxicillin + CLA[b]	0.75	0.75	12	8	8	2	1.5
Cephalothin	8	8	≥256	≥256	≥256	32	48
Cefotaxime	2	0.125	8	6	6	2	2
Ceftazidime	2	0.064	2	2	2	3	1
Cefepime	0.5	0.032	2	1.5	2	1.5	0.38
Aztreonam	3	0.094	6	6	12	12	3
Doripenem	0.094	0.032	0.19	0.19	0.19	0.25	0.094
Ertapenem	1.5	0.016	3	2	3	6	1
Imipenem	0.125	0.032	0.125	0.19	0.19	0.19	0.094
Meropenem	0.125	0.016	0.38	0.19	0.25	0.38	0.094

[a] MIC determined by Etest.
[b] CLA, clavulanic acid.

100985 (GenBank accession number ZP_09221116) and 96% with an open reading frame for a class D oxacillinase of *A. venetianus* VE-C3 (GenBank assembly ID, GCA_000308235.1). OXAs of VE-C3 and NBRC 100985 shared 97% identity. The sequences of the *rpoB* genes of NBRC 100985, CIP 110063^T, and VE-C3 were nearly identical (98.2 to 99.2% identity).

OXA-304 and OXA-305 from gen. spp. "Between 1 and 3" NIPH 542 and NIPH 817, respectively, were 98% identical with the class D β-lactamase from *Acinetobacter* sp. DR1 (GenBank accession no. YP_003732476).

The other OXAs exhibited from 57 to 77% identity with OXA-51.

Presence of oxacillinase genes in *Acinetobacter* species. We attempted to determine if the newly detected OXA-encoding genes were intrinsic to the various species. Unfortunately, for 15 species/taxa, only a single strain was available (Table 1). For all the 18 species/taxa with two or more strains in which a bla_{OXA} gene was found, all the members of the species/taxon possessed this, or a very closely related, gene with the exception of *A. parvus*. Within a species/taxon, the bla_{OXA} deduced sequences exhibited from 93 to 100% identity. Conversely, no bla_{OXA} genes were detected in the members of 13 species/taxa.

Of the eight *A. parvus* strains, five possessed an identical oxacillinase gene, whereas it was not detected in three strains, suggesting that $bla_{OXA-279}$ was lost or is not intrinsic to *A. parvus*.

A gene homologous to $bla_{OXA-272}$-$bla_{OXA-273}$, detected in the two *A. pittii* strains, was also present in the published genomes of *A. pittii* SH024, DSM 9306, DSM 21653, and D499 (GenBank accession numbers ADCH01000007, AIEF01000162, AIEK01000060, and AGFH01000023, respectively), indicating that the $bla_{OXA-272}$-$bla_{OXA-273}$-like gene is intrinsic to *A. pittii*.

A gene homologous to $bla_{OXA-268}$, found in *A. calcoaceticus*, was detected in the published genomes of two other *A. calcoaceticus* strains, RUH 2202 and PHEA-2 (GenBank accession numbers NZ_GG704949 and CP002177) and a homologue to $bla_{OXA-280}$-$bla_{OXA-281}$ was found in *A. johnsonii* SH046 (GenBank accession number NZ_GG704972). These two enzymes appear therefore to be intrinsic to their respective species.

All *A. baumannii* harbored a $bla_{OXA-51-like}$ gene. We found six new variants of $bla_{OXA-51-like}$ genes with deduced sequences from 94% to 99% identical to those of oxacillinases belonging to the OXA-51 group.

Taken together, these results indicate that several *Acinetobacter* species, such as *A. baumannii*, *A. pittii*, *A. calcoaceticus*, and *A. lwoffii* likely possess an intrinsic gene for a class D β-lactamase. For the other species, the number of isolates studied was too low to allow any conclusion.

Classification in OXA groups. Until now, as already mentioned, CHDLs have been assigned to five groups, OXA-23, OXA-24/40, OXA-51, OXA-58, and OXA-134, that exhibit lower than 80% mutual identity. Identities between each group as well as identities between OXAs belonging to the same group are indicated in Table 3. A phylogenetic tree was drawn from the OXA sequences (Fig. 1). Postulating that intragroup identity should be higher than 80% (Table 3), comparative analysis of the sequences of all OXA enzymes allowed to increase the number of OXA groups from 7 to 18 (Table 3 and Fig. 1). These data show a large diversity among class D β-lactamases since identity between OXAs ranged from 27% to 99%. OXA-2 and OXA-10 were distantly related, with 27% to 35% and 32% to 37% identity, respectively, with the other groups. The OXA-23, OXA-51, OXA-211, and OXA-279 groups were the most homogeneous (intragroup identity, >93%), whereas the intergroup identity varied from 49% to 77% (Table 3). Of the 11 new OXA groups, 8 groups (OXA-214, -286, -294, -279, -266, -299, -211, and -213) exhibited 58 to 61% identity with OXA-51. OXA-308 (from *A. gerneri*) displayed 54 to 55% identity with both OXA-51 and OXA-58 groups. Although OXA-274/275, OXA-300/301, and OXA-228 (all forming the so-called OXA-228 group) had greater than 81% identity, the percentages of identity between OXA-274/275 and OXA-51 and OXA-58 groups were similar (56 to 57%) whereas OXA-228, OXA-300, and OXA-301 were more related to OXA-58 (59% identity) than to OXA-51 (56% identity). Thus, one can hypothesize that bla_{OXA-58} and bla_{OXA-51} have probably diverged in parallel with the *A. guillouiae* and *A. bereziniae* species.

Comparison of OXA and RpoB phylogenetic trees. The phylogenetic tree obtained from comparative analysis of RpoB, deduced from the complete sequence of the *rpoB* gene of each strain of each species, is shown in Fig. 2. In order to compare this tree with that drawn from the OXA sequences, we have arbitrarily defined RpoB groups. Since RpoB sequences were at least 92% identical, and to be coherent with the RpoB phylogenetic tree, the cutoff percent identity was arbitrarily defined as 98%, allowing us to define 21 RpoB groups. The OXA and RpoB trees exhibited

similarities (see Fig. S2 in the supplemental material). With the exception of the OXA-134, OXA-213, OXA-228, and OXA-279 groups, all the OXAs forming a group were detected in species belonging to the same RpoB group (Table 4). The OXAs assigned to the OXA-134 group were detected in species of the RpoB groups 7 and 9, which are closely related, and those assigned to the OXA-213 group were found in species of the RpoB groups 1 and 2, which are also closely related. Similarly, OXA-228-type enzymes were found in two close species (*A. guillouiae* and *A. bereziniae*) (Fig. 2; see Fig. S2 in the supplemental material). The members of the OXA-279 group were detected in *A. parvus* (RpoB group 3) and in gen. sp. 6 (RpoB group 5) (Fig. 2). It is possible that *A. parvus* acquired OXA-279 from gen. sp. 6, which is coherent with the observation that three of eight *A. parvus* isolates did not harbor this bla_{OXA} gene.

To ensure that the result of RpoB-based phylogenetic analysis reflects also the phylogenies of other parts of the genome, we constructed a phylogenetic tree based on the concatenated partial sequences of seven housekeeping protein-coding genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*), which are found at distant loci of the *Acinetobacter* genome. As shown in Fig. S3 in the supplemental material, there were no significant differences between the resulting phylogenetic tree and that based on the RpoB sequences.

These data are consistent with the notion of the coevolution of the bla_{OXA} genes and of the host genome and thus ancient acquisition of almost all bla_{OXA} genes by *Acinetobacter* species. The other oxacillinases appeared to be species specific, and no intrageneric or intragroup transfer of oxacillinases seems to have occurred.

Origin of carbapenem resistance genes. It has been hypothesized that *A. radioresistens* is the source of bla_{OXA-23} for *A. baumannii* (23). To find a putative origin for genes $bla_{OXA-40/-143}$ and bla_{OXA-58}, we screened for their presence in species other than *A. baumannii*, but no genes closely related were found. Thus, the reservoir of these CHDL remains unknown. Screening for the presence of these three, or closely related, genes in the chromosomes of other bacterial genera available in GenBank database was also unsuccessful.

ADC in *Acinetobacter* spp. To date, ADC, the chromosomally encoded *Acinetobacter*-derived cephalosporinase, has been detected in a few *Acinetobacter* species, such as *A. baumannii*, *A. pittii*, and *A. baylyi* (20). Our analysis of 103 genomes revealed that a complete bla_{ADC}-like gene was present in 13 validly named species (*A. baumannii*, *A. baylyi*, *A. beijerinckii*, *A. bereziniae*, *A. calcoaceticus*, *A. guillouiae*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. nosocomialis*, *A. pittii*, *A. ursingii*, and *A. venetianus*), eight genomic species (gen. spp. 6, 13BJ/14TU, 14BJ, 15TU, 16, 17, "Close to 13TU," and "Between 1 and 3"), six taxa (taxa 18, 19, 20, 21, 22, and 26), and in the *A. pittii*-like and *A. calcoaceticus*-like strains (Table 1). The sequences of 65 new ADCs were from 34 to 99% identical. The phylogenetic tree obtained from the ADC sequences (Fig. 3) indicated that these cephalosporinases were separated in five main clusters (a cluster being composed of enzymes exhibiting more than 74% identity): one composed of ADC derived from the species and strains of the ACB complex (group 1) and the others, more heterogeneous, containing ADC from the other species (groups 2 to 5) (Fig. 3). Again, as observed for bla_{OXA}, bla_{ADC} was found in all the members of a given species, with the exception of *A. johnsonii* and *A. pittii*, in which only one

TABLE 6 Susceptibility to β-lactams of *E. coli* harboring cloned bla_{OXA} genes

Antibiotic	MIC (mg/liter) for *E. coli* TOP10 harboring:								
	No pCRblunt	pCRblunt::$bla_{OXA-279}$	pCRblunt::$bla_{OXA-290}$	pCRblunt::$bla_{OXA-273}$	pCRblunt::$bla_{OXA-270}$	pCRblunt::$bla_{OXA-304}$	pCRblunt::$bla_{OXA-301}$	pCRblunt::$bla_{OXA-266}$
Amoxicillin	6	≥256	64	32	8	24	≥256	64	16
Amoxicillin + CLA[a]	6	32	24	16	6	16	24	16	4
Cephalothin	4	6	3	4	3	3	6	4	0.064
Cefotaxime	0.047	0.064	0.094	0.047	0.047	0.047	0.094	0.064	0.25
Ceftazidime	0.25	0.38	0.25	0.25	0.25	0.25	0.25	0.25	0.047
Cefepime	0.047	0.064	0.047	0.047	0.047	0.047	0.047	0.047	0.094
Aztreonam	0.094	0.125	0.094	0.094	0.047	0.064	0.125	0.094	0.032
Doripenem	0.023	0.125	0.032	0.064	0.023	0.047	0.094	0.032	0.047
Ertapenem	0.006	0.19	0.047	0.064	0.006	0.012	0.047	0.047	0.38
Imipenem	0.25	0.75	0.38	0.50	0.25	0.25	0.25	0.38	0.032
Meropenem	0.023	0.094	0.032	0.064	0.023	0.023	0.032	0.032	0.032

[a] CLA, clavulanic acid.

TABLE 7 Antibiotic susceptibility of recombinant *A. baumannii*

Antibiotic	MIC (mg/liter) for strain:			
	BM4587	BM4587(pCMΩbla_{OXA-23})	BM4587(pCMΩ$bla_{OXA-279}$)	BM4587(pCMΩ$bla_{OXA-290}$)
Amoxicillin	8	≥256	16	16
Amoxicillin + CLA[a]	8	128	12	12
Cephalothin	≥256	≥256	≥256	≥256
Cefotaxime	3	8	3	4
Ceftazidime	1	2	1	1
Cefepime	0.75	6	0.75	0.5
Aztreonam	6	6	6	6
Doripenem	1.5	24	0.75	0.75
Ertapenem	1.5	>32	4	4
Imipenem	0.38	>32	0.5	0.5
Meropenem	0.25	16	1.5	1.5

[a] CLA, clavulanic acid.

of the two strains possessed the gene. These results indicate that bla_{ADC} is an intrinsic species-specific gene.

Among the 26 strains that did not possess a bla_{OXA} gene, 10 had no bla_{ADC} gene (Fig. 2). Thus, three species (*A. brisouii*, *A. soli*, and *A. towneri*) and two taxa (23 and 25) harbored neither an oxacillinase nor a cephalosporinase gene (Table 1 and Fig. 2).

Antibiotic susceptibility. The β-lactam susceptibility of seven strains harboring a new oxacillinase, *A. pittii* ANC 3678 (OXA-273), *A. pittii*-like ANC 4050 (OXA-270), gen. sp. "Between 1 and 3" NIPH 542 (OXA-304), gen. sp. 6 CIP A165 (OXA-290), *A. parvus* CIP 108168^T (OXA-279), *A. bereziniae* CIP 70.12^T (OXA-301), and *A. venetianus* CIP 110063^T (OXA-266), was studied. These strains were chosen since (i) OXA-270, OXA-273, and OXA-304 belonged to the OXA-213 group, which is the most related to the OXA-51 group; (ii) as postulated above, *A. parvus* had possibly acquired the oxacillinase from gen. sp. 6; and (iii) OXA-301 and OXA-266 belonged to groups distantly related to the OXA-51 group. The MICs of various β-lactams, including carbapenems, against these strains are listed in Table 5. *A. pittii* ANC 3678, *A. pittii*-like ANC 4050, and gen. sp. "Between 1 and 3" NIPH 542 were low-level resistant to amoxicillin, whereas *A. bereziniae* CIP 70.12^T, *A. venetianus* CIP 110063^T, *A. parvus* CIP 108168^T, and gen. sp. 6 CIP A165 exhibited only diminished susceptibility to this antibiotic, and the MICs were reduced in the presence of clavulanic acid. Resistance to cephalothin (MICs from 32 to ≥256 mg/liter) in *A. pittii* ANC 3678, *A. pittii*-like ANC 4050, *A. venetianus* CIP 110063^T, and *A. bereziniae* CIP 70.12^T could be due to the presence of an ADC, since these strains possessed a bla_{ADC} gene. No other *bla* genes were detected in the four strains. Gen. sp. 6 CIP A165 and *A. parvus* CIP 108168^T had similar cephalothin MICs despite the fact that CIP A165 harbored a bla_{ADC}, in contrast to CIP 108168^T. The seven strains were susceptible to third-generation (cefotaxime, ceftazidime) and fourth-generation (cefepime) cephalosporins as well as to all carbapenems. The bla_{OXA} genes were cloned using specific primers (Table 2) in plasmid pCRblunt and transformed into *E. coli* TOP10. For the transformants, the MICs of amoxicillin ranged from 8 mg/liter (for OXA-270 from *A. pittii*) to more than 256 mg/liter (for OXA-279 and OXA-301 from *A. parvus* and *A. bereziniae*, respectively) and an MIC decrease, from 1.5- to more than 10-fold, was observed in the presence of clavulanic acid (Table 6). MICs of cephalosporins were not affected by the presence of the bla_{OXA} genes. OXA-279 (from *A. parvus*) was responsible for increases of 3-, 4-, 5-, and 32-fold of the MICs of imipenem, meropenem, doripenem, and ertapenem, respectively. OXA-273 (from *A. pittii*) led to 2-, 3-, 3-, and 10-fold increases of the level of resistance to imipenem, doripenem, meropenem, and ertapenem, respectively (Table 6). The genes for OXA-279 (*A. parvus*) and OXA-290 (gen. sp. 6), were cloned in pCM88 and electrotransformed into susceptible *A. baumannii* BM4587 (30). Strain BM4587 harboring bla_{OXA-23} cloned in pCM88 was used as a positive control (Table 7). Whereas BM4587(pCM88) was clearly resistant to carbapenems, a very weak decrease in susceptibility of BM4587(pCM88Δbla_{OXA-23}Ω$bla_{OXA-279}$) and BM4587(pCM88Δbla_{OXA-23}Ω$bla_{OXA-290}$) was observed for meropenem (5-fold), imipenem (1.5-fold), and ertapenem (2.5-fold), indicating that these oxacillinases have only a weak activity against carbapenems.

As previously mentioned, the 21 *A. baumannii* strains included in this study possessed the bla_{ADC} and $bla_{OXA-51-like}$ genes. Six strains had at least an additional β-lactamase, and three strains, ANC 4097, NIPH 1734, and NIPH 1362, were resistant to carbapenems (Table 8). The highly imipenem-resistant ANC 4097 strain possessed bla_{OXA-69}, bla_{OXA-23}, bla_{TEM-1}, and bla_{NDM-1} (41), whereas $bla_{OXA-51-like}$ and bla_{OXA-58} were identified in NIPH 1734. NIPH 1362 carried a $bla_{OXA-51-like}$ gene with an IS*Aba1* located 79 bp upstream, which could explain, as reported previously (19), the diminished susceptibility to imipenem (MIC, 16 mg/liter) of this strain. NIPH 2061 exhibited diminished susceptibility to imipenem (MIC, 4 mg/liter) with neither IS*Aba1* nor IS*Aba9* upstream of bla_{OXA-51}. Four strains (NIPH 1669, NIPH 601, NIPH 70, and CIP 70.34^T) had a weak increase in imipenem MIC (MIC, 1 mg/liter). For the 13 remaining strains, the MICs of imipenem ranged from 0.25 to 0.5 mg/liter. Thus, similarly to bla_{OXA-51} and its derivatives described to date, the new $bla_{OXA-51-like}$ seems to have only a moderate effect on the activity of carbapenems.

In conclusion, our study allowed the identification of 50 new oxacillinases and of 65 new ADC cephalosporinases, which indicates the high prevalence and diversity of these β-lactamases in the genus *Acinetobacter*. Based on phylogenetic analysis of amino acid sequences deduced from bla_{OXA} sequences, the oxacillinases could be classified into 18 groups. Except for the four OXAs related to OXA-228, all the new enzymes were more related to the OXA-51 and OXA-134 groups than to the OXA-23, -24/40, and -58 groups. Acquisition of the oxacillinase genes is likely to be an ancient event in *Acinetobacter* spp. since almost all of the new OXAs are species

TABLE 8 β-Lactam susceptibility and β-lactamase content of 21 *A. baumannii* strains[a]

Strain	MIC (μg/liter)							β-Lactamase gene(s)
	TIC	TCC	CF	FOX	CAZ	IMP	ETP	
ANC 4097	256	256	128	256	256	64	256	IS*AbaI*-bla_{OXA-69} + IS*AbaI*-bla_{OXA-23} + bla_{TEM-1} + bla_{NDM-1}
NIPH 1734	256	256	128	256	4	32	256	$bla_{OXA-51-like}$ + bla_{OXA-58}
NIPH 1362	256	256	128	256	256	16	256	IS*AbaI*-$bla_{OXA-51-like}$
NIPH 24	256	64	128	128	4	0.5	4	bla_{OXA-66} + bla_{TEM-1}
NIPH 528	256	64	128	128	2	0.5	2	bla_{OXA-66} + bla_{TEM-1}
NIPH 527	256	32	128	128	4	0.5	4	bla_{OXA-69} + bla_{TEM-1}
NIPH 290	256	16	128	64	64	0.5	2	bla_{OXA-69} + bla_{TEM-1}
NIPH 1669	64	128	128	256	32	1	64	bla_{OXA-71}
NIPH 2061	64	32	128	256	256	4	256	$bla_{OXA-109}$
CIP 70.34^T	16	8	128	256	8	1	16	bla_{OXA-98}
NIPH 335	16	8	128	128	16	0.5	4	$bla_{OXA-128}$
NIPH 601	8	2	128	32	1	1	0.5	bla_{OXA-69}
NIPH 67	4	4	128	128	4	0.5	4	$bla_{OXA-51-like}$
NIPH 70	4	2	128	64	2	1	4	$bla_{OXA-51-like}$
NIPH 80	4	2	128	256	4	0.5	16	bla_{OXA-78}
NIPH 201	2	2	128	64	2	0.5	2	$bla_{OXA-51-like}$
NIPH 329	1	1	128	128	2	0.5	4	$bla_{OXA-51-like}$
NIPH 60	1	1	128	64	2	0.25	2	bla_{OXA-69}
NIPH 146	1	1	128	64	1	0.25	2	bla_{OXA-64}
NIPH 190	0.5	0.25	128	16	0.5	0.5	1	bla_{OXA-65}
NIPH 615	0.25	0.25	128	32	0.5	0.5	1	$bla_{OXA-51-like}$

[a] Abbreviations: CAZ, ceftazidime; CF, cephalothin; ETP, ertapenem; FOX, cefoxitin; IMP, imipenem; TCC, ticarcillin plus clavulanic acid; TIC, ticarcillin.

specific. No indication of an interspecific bla_{OXA} transfer between distantly related species was observed, except between gen. sp. 6 and *A. parvus*. Since resistance genes in *Acinetobacter* can be transferred via plasmids and/or transposons by conjugation or transformation, these new enzymes represent a large pool of oxacillinases that are potentially transferable to pathogenic *Acinetobacter* species, such as *A. baumannii*. However, all bacterial hosts were susceptible to carbapenems and the few oxacillinases tested in this study exhibited poor activity against these drugs. The ADC class C β-lactamases in *A. baumannii* and the other members of the ACB complex was very conserved but only distantly related to those in other species, which is consistent with the absence of horizontal transfer of these chromosomal enzymes among *Acinetobacter* spp.

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